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Regulation of cortical dendrite development by Rap1 signaling

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Rap1 is a small GTP-binding protein that has been implicated in intracellular signaling and cytoskeletal control. Here, we show that Rap1 is expressed in rat cortical neurons and plays a critical role in dendritic development. Inhibition of Rap1 signaling either by expressing dominant negative mutant of Rap1 or Rap1GAP in cortical neurons reduced dendritic complexity. In contrast, expression of a constitutively active mutant of Rap1 (Rap1V12) induced dendritic growth and branching. Membrane depolarization, which induces dendritic growth via calcium influx, led to a rapid activation of Rap1 via cAMP and cGMP signaling. A CREB-dependent mechanism is involved in depolarization-induced dendritic growth in cortical neurons. Rap1 function contributed to depolarization induced CREB activation, and inhibition of CREB suppressed dendritic growth induced by Rap1V12. These observations identify Rap1 as a key mediator of calcium regulation of CREB-dependent transcription and dendritic development.

Introduction

Dendritic morphology is a key determinant of neuronal connectivity. The development of the dendritic tree is regulated by both activity-dependent and activity-independent mechanisms (Jan and Jan, 2003; Wong and Ghosh, 2002). The effects of neuronal activity on dendritic development are mediated by intracellular calcium signals (Cline, 2001; Miller and Kaplan, 2003; Wong and Ghosh, 2002). It is likely that calcium influx regulates dendritic morphogenesis by controlling the activation or expression of proteins that affect the neuronal cytoskeleton. An example of this mechanism is the calcium activation of Rho-family GTPases, which regulates various aspects of actin cytoskeleton (Hall and Nobes, 2000). The Rho family proteins Rac and Cdc42 are activated by calcium entry and they exert a positive effect on dendritic growth (Li et al., 2002; Luo et al., 1996; Sin et al., 2002; Threadgill et al., 1997; Wong et al., 2000).

While the Rho-family GTPases exert their cytoskeletal effects mostly by local control of the actin cytoskeleton, there is emerging evidence that calcium signaling to the nucleus also contributes to dendritic growth and branching. For example, calcium signaling to the cAMP-responsive element binding protein (CREB) is required for calcium-dependent dendritic growth in cortical neurons (Redmond et al., 2002). Calcium induction of CREB-dependent transcription occurs via activation of the CaM kinase (CaMK) and extracellular signal regulated-protein kinase (ERK) pathways, and inhibitors of both CaMK and ERK suppress depolarization-induced dendritic growth (Redmond et al., 2002; Vaillant et al., 2002). Thus, ERK- and CaMK-mediated induction of CREB-dependent transcription represents an important mechanism of dendritic growth in response to calcium influx.

While the mechanism by which calcium influx regulates CaMK activation is fairly well understood, several mechanisms have been proposed to account for calcium activation of ERK. Two likely mediators are the small GTP-binding proteins Ras and Rap. Both Ras and Rap are activated by calcium influx in non-neuronal cells (Franke et al., 1997; Grewal et al., 2000b; Rosen et al., 1994), and calcium activation of Ras has been demonstrated in cortical neurons (Rosen et al., 1994). The role of Ras and Rap in regulating dendritic growth and remodeling has not been explored, although these proteins have been implicated in cytoskeletal control in non-neuronal cells (Caron, 2003; Schmidt and Hall, 1998; Zwartkruis and Bos, 1999).

Just like other small GTPases, the activities of Rap are carefully regulated by activators, the guanine nucleotide exchange factors (GEFs), and inactivators, the GTPase-activating proteins (GAPs). Rap1 is likely to regulate dendritic development because a prominent function of Rap1 is the activation of integrin receptors, which has been implicated in neurite outgrowth and modulation of cytoskeleton (Caron, 2003; Clegg, 2000). Also, the ability of SPAR, a Rap1GAP, to cause spine head enlargement has been attributed to inhibition of Rap1 signaling in filopodia-like protrusions on dendritic shafts (Pak et al., 2001).

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In this study, we investigate the cellular function and calcium activation properties of Rap1 in cortical neurons. We report that Rap1 is a potent regulator of dendritic morphology, and is activated by calcium influx through *N*-methyl-D-aspartate (NMDA) receptors and voltage-sensitive calcium channels (VSCCs). We also show that Rap1 contributes to calcium activation of ERK1/2 and induction of CREB-dependent transcription. Furthermore, we provide evidence that Rap1 regulates dendrite morphogenesis, at least in part, via CREB-dependent gene expression.

Results

Localization of Rap1 in cortical neurons

To determine the distribution of Rap1 in cortical neurons, we carried out immunofluorescence studies using embryonic day 18 (E18) rat cortical cultures. We used a mouse monoclonal anti-Rap1 antibody that detected a single band at approximately 21 kDa, a molecular weight corresponding to that of Rap1, on immunoblots containing lysates of cortical neurons (Fig. 1a). Immunofluorescence analysis using this antibody revealed that Rap1 protein was present in the soma and also in dendrites of cortical neurons (Fig. 1b). In addition, double immunofluorescence studies using a rabbit polyclonal anti-Rap1 antibody along with a mouse monoclonal antibody against the somato-dendritic marker MAP2 revealed colocalization of Rap1 and MAP2 (Fig. 1c). Therefore, Rap1 is present in the cell body as well as dendritic processes of cortical neurons. We also examined Rap1 expression in the developing postnatal cortex. Consistent with its proposed role in dendritic development, we found that Rap1 is expressed in the cortical plate (Supplementary Fig. 1).

Role of Rap1 in the development of cortical dendrites

To determine whether Rap1 function is required for proper dendritic development, we examined the effects of expressing dominant negative (Rap1N17-EGFP) and constitutively active (Rap1V12-EGFP) forms of Rap1 in cortical neurons (Pizon et al., 1999). Fusing Rap1N17 or Rap1V12 with EGFP as a single fusion protein allowed us to unambiguously identify the transfected neurons. Rap1N17 is believed to specifically affect Rap1 signaling without affecting the Ras pathway. For example, expression of Rap1N17 in the mouse forebrain has no effect on the amount of active Ras in the GTP-bound state (Morozov et al., 2003), and Rap1N17 expression does not block NGF activation of Ras (York et al., 1998).

We transfected E18 cortical cultures shortly after plating, and processed the cultures for immunofluorescence and morphometric analysis at 2 DIV. Inhibition of Rap1 function by Rap1N17-EGFP expression led to a marked decrease in dendritic complexity, and expression of constitutively active Rap1 (Rap1V12-EGFP) stimulated dendritic growth and branching (Fig. 2a). Results of Scholl analysis, which provides measures of dendritic length and branching patterns, confirmed this impression. In control EGFP-expressing neurons, there was a gradual decrease in the number of intersections with distance along the dendritic tree. Compared to EGFP expression, expression of Rap1N17-EGFP significantly decreased the number of dendrite crossings at 10 μm (33%), 20 μm (26%), and 30 μm (38%) distances from the cell body indicating that the loss of Rap1 function inhibits dendritic development (Fig. 2b). In addition to Rap1N17, we found that expression of another inhibitor of Rap, Rap1GAP, similarly decreased the dendritic complexity (discussed later in the text). In contrast, Rap1V12-EGFP expression significantly increased the

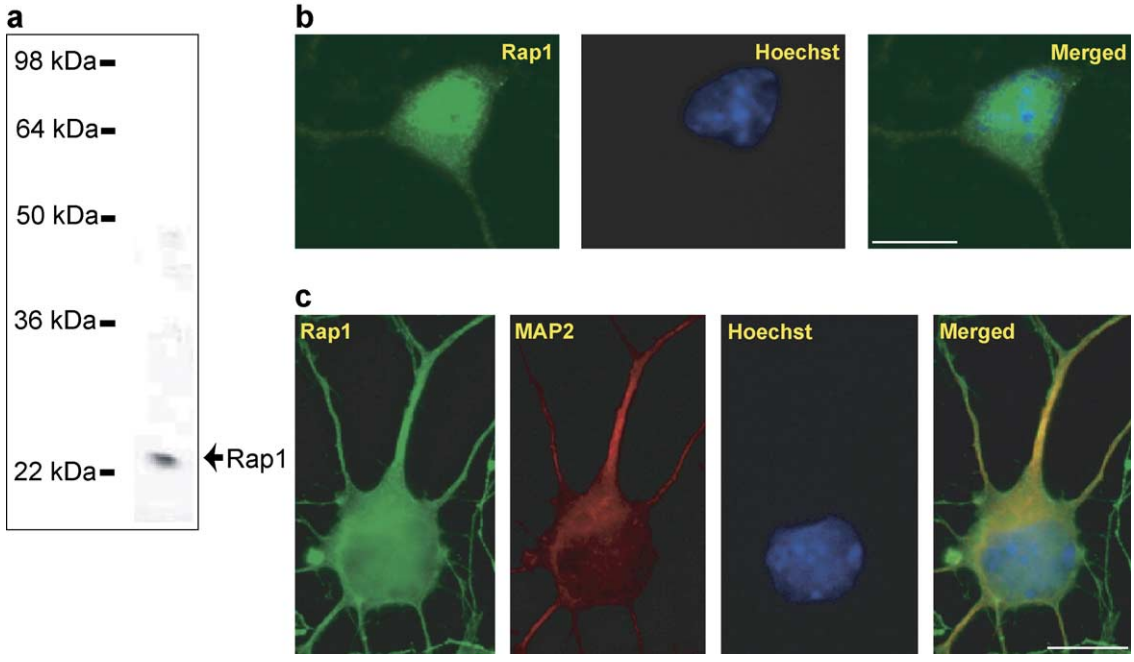


Fig. 1. Subcellular localization of Rap1 in cortical neurons. (a) A mouse monoclonal antibody against Rap1 detected only a single band at approximately 21 kDa in the immunoblot containing E18 cortical neuron lysates. (b) E18 cortical neurons were fixed 20–24 h after plating and stained for the endogenous Rap1 using the same monoclonal anti-Rap1 antibody used in (a). The images of a representative neuron are shown. (c) The images of a neuron double labeled with a polyclonal rabbit anti-Rap1 antibody and a mouse monoclonal anti-MAP2 antibody are shown. Scale bar, 10 μm.

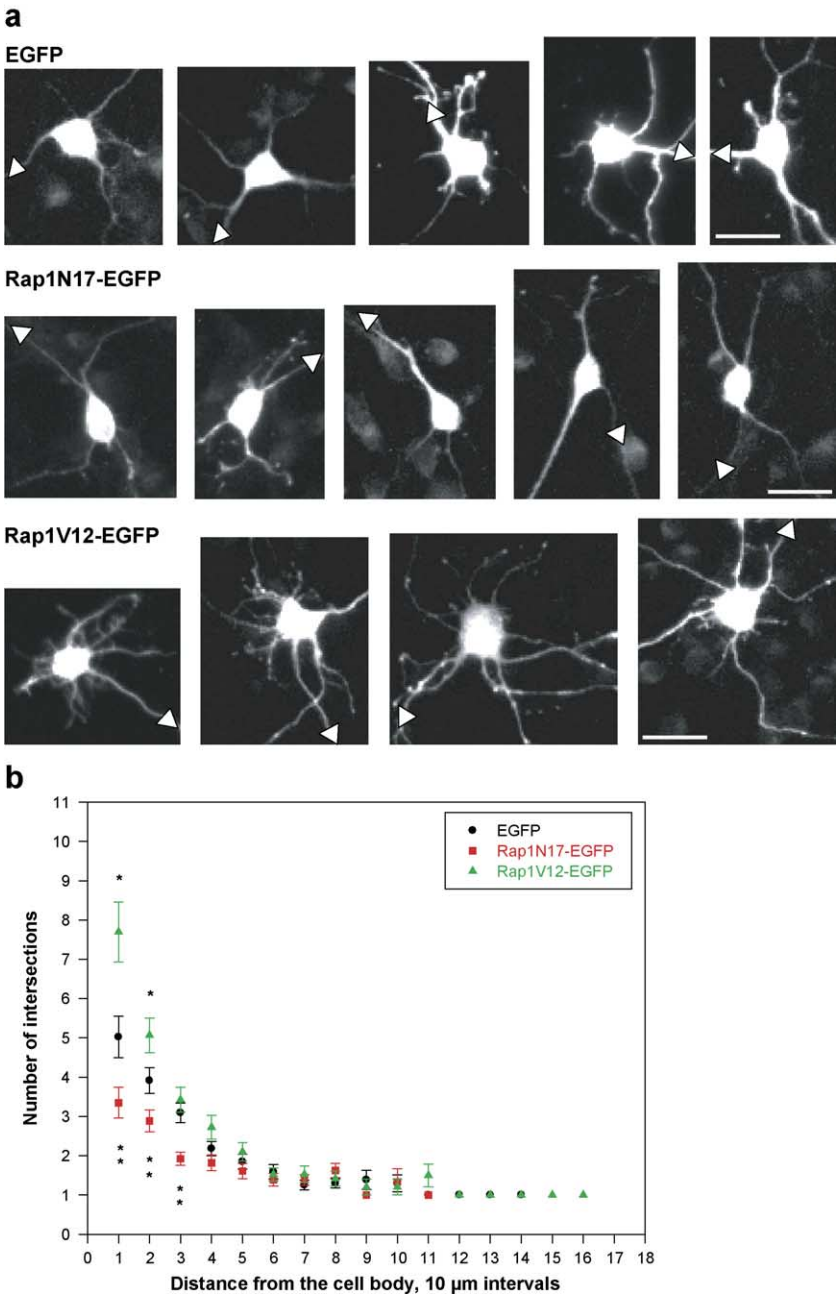


Fig. 2. Rap1 regulates dendritic development in cortical neurons. (a) Examples of dissociated E18 cortical neurons transfected with plasmids encoding EGFP, Rap1N17-EGFP and Rap1V12-EGFP. Transfected neurons were analyzed by immunofluorescence. Scale bar, 20 μm. (b) Scholl analysis of dendritic branches presented as mean number of intersections plotted as a function of distance from the cell body (center = 0). Typically, 15–20 neurons were analyzed per condition within each experiment. Data from three independent experiments are shown as mean ± SEM. Single asterisks indicate statistically significant differences ($p < 0.05$) between EGFP-expressing cells and Rap1V12-EGFP-expressing neurons by Student's t test. Double asterisks indicate statistical significance ($p < 0.05$) between EGFP-expressing cells and Rap1N17-EGFP-expressing neurons.

number of dendrite crossings at 10 μm (53%) and 20 μm (30%) distances. Thus, Rap1 signaling regulates dendritic morphology in cultured cortical neurons.

To determine whether Rap1 was involved in regulating dendritic development in a more physiological context, we decided to study the role of Rap1 in postnatal cortical slice cultures. The slice cultures retain many aspects of synaptic activity and cellular organization that are present in vivo (Redmond et al., 2002). We examined the effects of altering Rap1 function on the basal dendritic trees of layer 5 pyramidal neurons since these neurons develop extensive basal dendritic trees that are well-maintained in slice cultures. Interfering with the endogenous Rap1 function by expressing Rap1N17-EGFP resulted in a marked decrease in the complexity of layer 5 basal dendrites indicating that the endogenous Rap1 contributes to dendritic development in these neurons (Figs. 3a and b). Note that basal dendrites are shorter in length in Rap1N17-expressing neurons. Since Rap1N17 affected the complexity of neurons by reducing the length and the number of

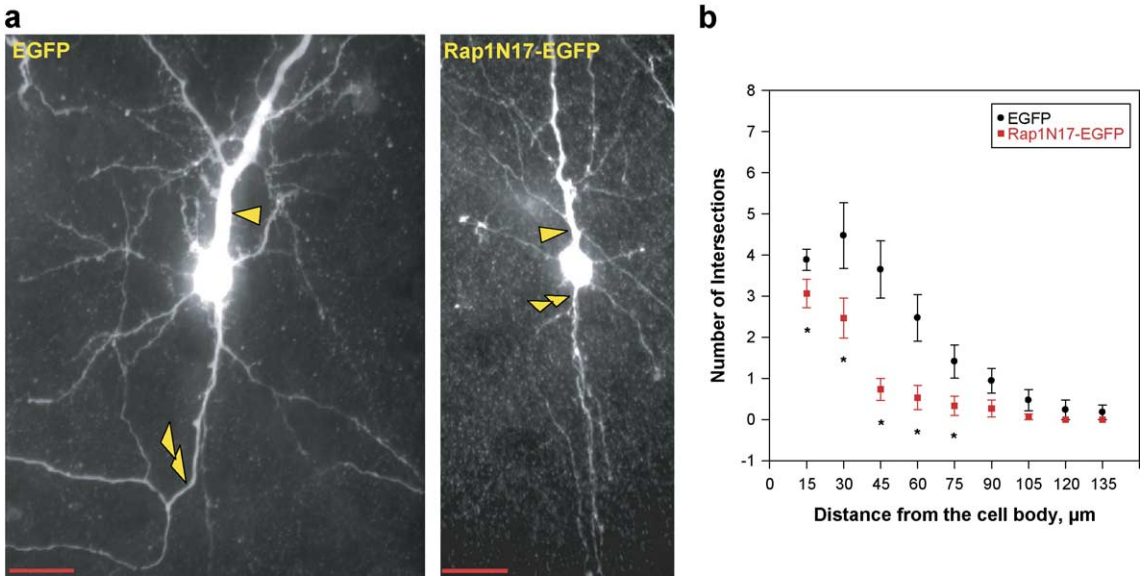


Fig. 3. Inhibition of the endogenous Rap1 function reduces dendritic growth and complexity of basal dendrites of layer 5 pyramidal neurons in cultured cortical slices. (a) Examples of basal dendritic trees of layer 5 pyramidal neurons transfected with EGFP or Rap1N17-EGFP encoding plasmid at P5 + 1 DIV and examined by immunofluorescence at P5 + 3 DIV. Single arrowheads identify the apical dendrites. Double arrowheads identify the axons. Scale bar, 20 μm. (b) Scholl analysis of basal dendritic tree of layer 5 pyramidal neurons transfected as indicated. Image stacks of a total of 15–18 neurons per condition collected from three independent experiments were reconstructed and analyzed. Results are presented as mean number of intersections plotted as a function of distance from the cell body (center = 0). Asterisks indicate statistical significance ($p < 0.05$) by Student's t test.

intersections, it appears that Rap1 signaling regulates outgrowth, lengthening, and branching of dendrites. Thus, based on the results of inhibiting Rap1 function in both dissociated neurons and cortical slices, we conclude that Rap1 plays a critical role in regulating the morphological development of cortical dendrites.

Calcium activation of Rap1 in cortical neurons

The finding that Rap1 regulates dendritic development raised the possibility that extracellular signals might control dendritic growth via Rap1. A number of signals including neurotrophins and calcium influx have been shown to exert a positive effect on cortical dendritic development (Miller and Kaplan, 2003; Wong and Ghosh, 2002). To determine whether these signals could activate Rap1 in cortical neurons, we performed "pull-down" assays, which involve the use of GST-tagged Rap-binding-domain of RalGDS (GST-RalGDS-RBD) to capture Rap1 in the GTP-bound state. In this assay, quantifying the amount of Rap1 that can be precipitated with GST-RalGDS-RBD provides a measure of activated Rap1 in cell lysates (Franke et al., 1997). Preliminary experiments showed that KCl-induced membrane depolarization, which leads to calcium entry, results in a much more robust activation of Rap1 compared to the other stimuli tested (data not shown). Therefore, in the remainder of the study, we focused on analyzing the calcium activation properties of Rap1 and investigating its role in calcium-dependent dendritic growth.

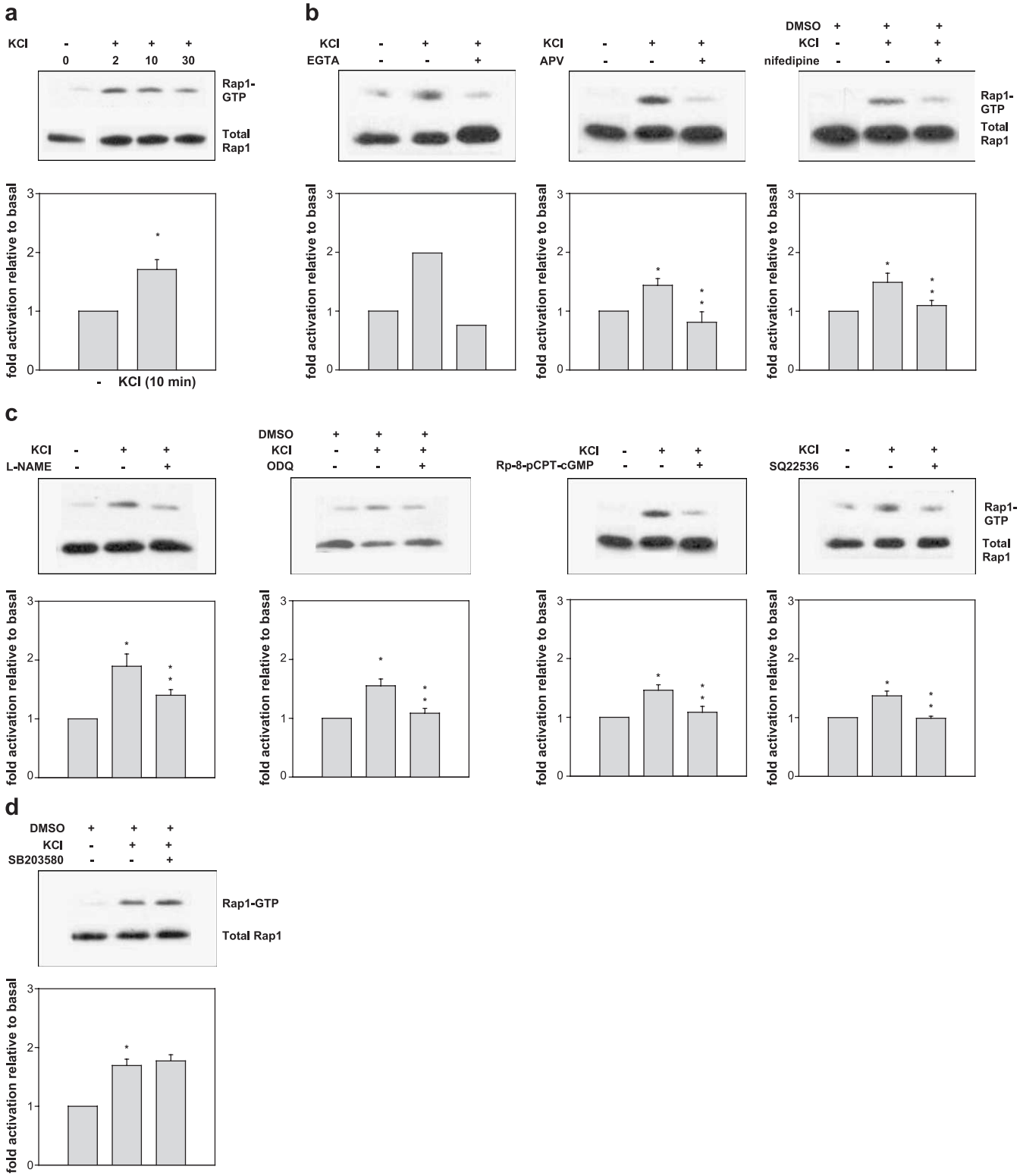
To determine the kinetics of Rap1 activation by membrane depolarization, we treated cultured neurons with 50 mM KCl and measured Rap1 activation 2, 10, and 30 min later. A rapid increase in the amount of active, GTP-bound Rap1 was detectable 2 min after depolarization. This activation was sustained for at least 30 min (Fig. 4a, top panel). Quantification of results showed that there was approximately 1.7 fold activation of Rap1 activity following KCl stimulation for 10 min (Fig. 4a, bottom panel). To determine whether other means of stimulating calcium entry such as activation of glutamate receptors could also lead to Rap1 activation, we treated cells with 100 μM glutamate. As with depolarization, glutamate stimulation resulted in a rapid activation of Rap1, which returned to near basal levels by 10 min (Supplementary Fig. 2). When cells were treated with the calcium chelator EGTA before KCl stimulation, KCl activation of Rap1 was abolished to near the basal level (Fig. 4b), indicating that calcium influx is necessary for Rap1 activation. In addition, APV (an NMDA receptor blocker) or nifedipine (an L-type VSCC blocker) treatment significantly inhibited KCl activation of Rap1

Fig. 4. Calcium influx regulates Rap1 activation via cGMP- and cAMP-mediated signaling cascades. Pull-down assays were used to measure Rap1 activity. The fraction of Rap1 in the active GTP-bound form was determined by comparing the amount of Rap1-GTP with that of total Rap1 protein. (a) Levels of activated Rap1 in neurons stimulated with KCl for 2, 10 and 30 min. In the top panel is a representative blot from multiple experiments. The bottom panel shows quantification results presented as fold activation of Rap1 following 10-min KCl stimulation relative to basal, unstimulated state. Asterisk indicates statistical significance ($p < 0.05$) using Student's t test. (b–d) The effects of various pharmacological inhibitors on KCl activation of Rap1. Cortical neurons were pretreated with EGTA ($n = 2$), APV ($n = 4$), nifedipine ($n = 3$), L-NAME ($n = 4$), ODQ ($n = 4$), Rp-8-pCPT-cGMP ($n = 5$), SQ22536 ($n = 4$) and SB203580 ($n = 3$) before 10-min KCl stimulation. The concentrations of inhibitors used are noted in Methods. Top panels show representative blots from multiple experiments. Bottom panels show quantification results as fold activation relative to basal, unstimulated state. Single asterisks indicate statistical significance ($p < 0.05$) between unstimulated cells and KCl-stimulated cells by Student's t test. Double asterisks indicate statistical significant differences ($p < 0.05$) between KCl-stimulated cells and inhibitor-treated KCl-stimulated cells.

(Fig. 4b). Therefore, calcium influx via both NMDA receptors and L-type VSCCs contributes to Rap1 activation.

Role of cGMP and cAMP signaling in calcium activation of Rap1

Since cGMP and cAMP are major effectors of calcium signaling, we decided to examine the role of these second messengers in calcium activation of Rap1. Calcium influx regulates cGMP levels via activation of calcium-calmodulin sensitive nitric oxide synthase (NOS) (Berridge et al., 2000). Activation of NOS results in the generation of NO, which activates soluble guanylyl cyclase (sGC) to increase cGMP levels. The biological effect of cGMP is mediated mostly by cGMP-dependent protein kinase (PKG). Calcium regulates cAMP



levels via activation of calcium-calmodulin sensitive adenylate cyclase (AC), which enhances cAMP production (Berridge et al., 2000).

Pharmacological perturbation experiments indicated that both the NO/cGMP- and cAMP-mediated pathways contribute to calcium activation of Rap1. As shown in Fig. 4c, the NOS inhibitor L-NAME, the sGC inhibitor ODQ, and the PKG inhibitor Rp-8-pCPT-cGMP, all attenuated KCl activation of Rap1. To determine whether activation of the cGMP-mediated pathway(s) alone can activate Rap1, we measured Rap1 activity in cells before and following treatment with a cell-permeable analog of cGMP, 8-pCPT-c-GMP. Our results showed that Rap1 was rapidly activated by 8-pCPT-c-GMP within 2 min (Supplementary Fig. 2). However, similar to glutamate treatment, this induction declined within 10 min.

The AC inhibitor, SQ22536, also suppressed KCl activation of Rap1 (Fig. 4c). This is consistent with prior results showing Rap1 activation by cAMP signaling (Vossler et al., 1997). The cAMP-dependent, calcium activation of Rap1 is likely mediated by a class of cAMP-binding Rap1GEFs (cAMP-GEF/Epac) or PKA. We attempted to determine whether PKA is involved in cAMP-mediated, calcium activation of Rap1 by using three different PKA inhibitors that act via distinct mechanisms. These inhibitors are Rp-cAMP (a non-hydrolyzable competitive cAMP analog inhibitor of PKA), 5-24 (a synthetic peptide substrate that inhibits PKA by binding to the catalytic site), and H89 (an inhibitor that blocks the kinase activity by competing against ATP but not the cAMP binding). While H89 (10 μM) effectively blocked KCl activation of Rap1 as previously reported in PC12 cells (Grewal et al., 2000b), Rp-cAMP (100 μM) and 5–24 (10 μM) only caused a minor reduction (data not shown). Although H89 is commonly used to block PKA activity, it does exhibit a weak inhibitory action against other kinases such as PKG, PKC, casein kinase I and II, myosin light chain kinase, and CaMK II. It is possible that the previously reported strong inhibitory effect of H89 on Rap1 activation might have resulted from its non-specific effects on other kinases. Therefore, based on these results, we cannot formally confirm the involvement of PKA in calcium-induced, cAMP-dependent Rap1 activation.

A previous study had implicated p38 kinase acting upstream of Rap1 (Schmidt et al., 2001). However, we found that the p38-specific inhibitor, SB203580, had no significant effect on KCl activation of Rap1 (Fig. 4d) indicating that p38 is not involved in calcium activation of Rap1. Together, our results support that cGMP- and cAMP-mediated signaling pathways are necessary for depolarization-induced Rap1 activation.

Role of Rap1 in depolarization-induced dendritic growth

The ability of calcium influx to regulate Rap1 activation and the fact that Rap1 signaling controls dendritic development led us to investigate whether calcium-dependent dendritic growth involves Rap1 signaling. To determine if Rap1 signaling was required for depolarization-induced dendritic growth, we transfected E18 cortical neurons with constructs encoding EGFP and Rap1N17-EGFP and examined the ability of transfected neurons to extend dendrites in response to KCl-induced depolarization. Consistent with previous results (Redmond et al., 2002), membrane depolarization caused a significant increase in dendritic complexity as reflected by an increase in the number of intersections at 10 and 20 μm distances from the soma (Fig. 5a). This effect was suppressed by Rap1N17-EGFP expression indicating that inhibiting Rap1 suppresses dendritic growth induced by calcium influx.

As an alternate way to examine the role of Rap1 in calcium-induced dendritic growth, we determined the effect of expressing the Rap1 inhibitor, Rap1GAP. Rap1GAP does not share homology with RasGAP, RanGAP, or RhoGAP, and is considered to be a Rap-specific GAP. In further support of its specificity, Rap1GAP has been shown to be inactive towards Ras, Rho, Cdc42 and Rac GTPases (Polakis et al., 1991). As shown in Fig. 5b, expression of Rap1GAP also suppressed KCl-induced dendritic growth. Thus, Rap1 appears to be a critical mediator of depolarization-induced dendritic growth.

We also examined the contribution of Rap1 in depolarization-induced dendritic growth in cortical slice cultures. Membrane depolarization led to a marked increase in dendritic complexity in layer 5 pyramidal neurons in slice cultures (Fig. 5c). However, Rap1N17-EGFP expression significantly reduced the dendritic complexity of KCl-treated pyramidal neurons to approximately that of untreated, Rap1N17-EGFP-expressing cells (Fig. 5c). Thus, as in the case of dissociated cell cultures, Rap1N17-EGFP expression suppressed the effect of membrane depolarization on dendritic growth. Taken together, our observations indicate that depolarization-induced dendritic growth and branching require Rap1 function.

Role of Rap1 in calcium activation of CREB

We have previously shown that calcium regulation of dendritic development in cortical neurons requires CREB-dependent transcription (Redmond et al., 2002). Both CaMK and ERK signaling pathways contribute to calcium-dependent phosphorylation and activation of CREB (Deisseroth et al., 1998; Dudek and Fields, 2002; Enslen et al., 1996; Hardingham et al., 2001; Impey et al., 1998; Sheng et al., 1991; West et al., 2002). Since Rap1 signaling has been implicated in ERK activation in non-neuronal cells (Stork, 2003), in the final series of experiments, we decided to test if Rap1 is involved in calcium activation of ERKs and CREB. We also examined whether ERK activity and CREB-dependent transcription are required for Rap1 function in dendritic growth.

To determine whether Rap1 could mediate calcium activation of ERKs in cortical neurons, we measured ERK1/2 phosphorylation levels following KCl treatment in EGFP- or Rap1N17-EGFP-expressing cells using antibodies that detect phosphorylated ERK1/2. KCl stimulation led to a significant increase in ERK1/2 phosphorylation within 10 min (Figs. 6a and b). However, this induction was suppressed in cells expressing Rap1N17-EGFP, indicating that Rap1 function is required for calcium-induced ERK phosphorylation. In addition, we found that APV, nifedipine, EDTA, SQ22536, L-NAME, Rp-8-pCP5T-cGMP, and ODQ, which prevented KCl activation of Rap1 (Figs. 4b and c), partially inhibited ERK phosphorylation (Supplementary Fig. 3). By contrast, SB203580 had no effect on KCl activation of either Rap1 (Fig. 4d) or ERK (Supplementary Fig. 3b). These results are consistent with the idea of ERK acting downstream of Rap1 signaling.

We next examined the involvement of Rap1 in calcium activation of CREB. Activation of CREB involves phosphorylation at Ser-133. Both CaMK IV- and ERK1/2-mediated pathways have been implicated in CREB phosphorylation and activation. It is thought that CaMK IV mediates rapid induction of CREB phosphorylation and ERK1/2 is required to sustain CREB phosphorylation (Dolmetsch et al., 2001; Ho et al., 2000; Ribar et

al., 2000; Wu et al., 2001b). We used a phospho-specific antibody to monitor CREB phosphorylation following KCl stimulation in cells expressing either EGFP or Rap1N17-EGFP. As previously reported, depolarization rapidly induced CREB phosphorylation at Ser-133 (2.46 ± 0.23 fold induction 2 min after KCl stimulation; $n = 4$) in EGFP expressing cells. This induction was sustained for at least 30 min (Fig. 6c). Although KCl induction of CREB phosphorylation was not significantly affected by Rap1N17-EGFP expression within the initial 10 min, it declined between 10 to 30 min following stimulation. Quantification revealed that 30 min following KCl stimulation, the levels of KCl-induced CREB phosphorylation in Rap1N17-EGFP-expressing cells was 68% of that of EGFP-expressing cells (Fig. 6d). Thus, Rap1 activity is necessary to sustain high levels of CREB phosphorylation following calcium influx, and this high levels of CREB phosphorylation is believed to be critical for transcription (Wu et al., 2001b).

To determine whether Rap1 contributes to calcium activation of CREB/CBP-mediated transcription, we examined the effects of Rap1GAP expression on the abilities of Gal4-CREB and Gal4-CBP to transactivate a UAS-CAT reporter in the presence or absence of KCl. As previously described (Sheng et al., 1991), KCl stimulation led to a robust activation of Gal4-CREB resulting in enhanced CAT activity (Fig. 6e). Expression of Rap1GAP reduced KCl-induced CAT activity by 40% indicating that Rap1 signaling is involved in calcium induction of CREB-dependent transcription. As shown in Fig. 6f, Rap1GAP expression also significantly inhibited the ability of KCl to transactivate Gal4-CBP. Thus, Rap1 signaling contributes to CREB- and CBP-mediated transcription.

Calcium induction of the c-*fos* promoter, which contains a CRE sequence, is known to be mediated by ERK signaling to CREB and CBP. Since Rap1 is involved in the activation of ERK1/2, CREB, and CBP, we next tested whether Rap1 function was necessary for

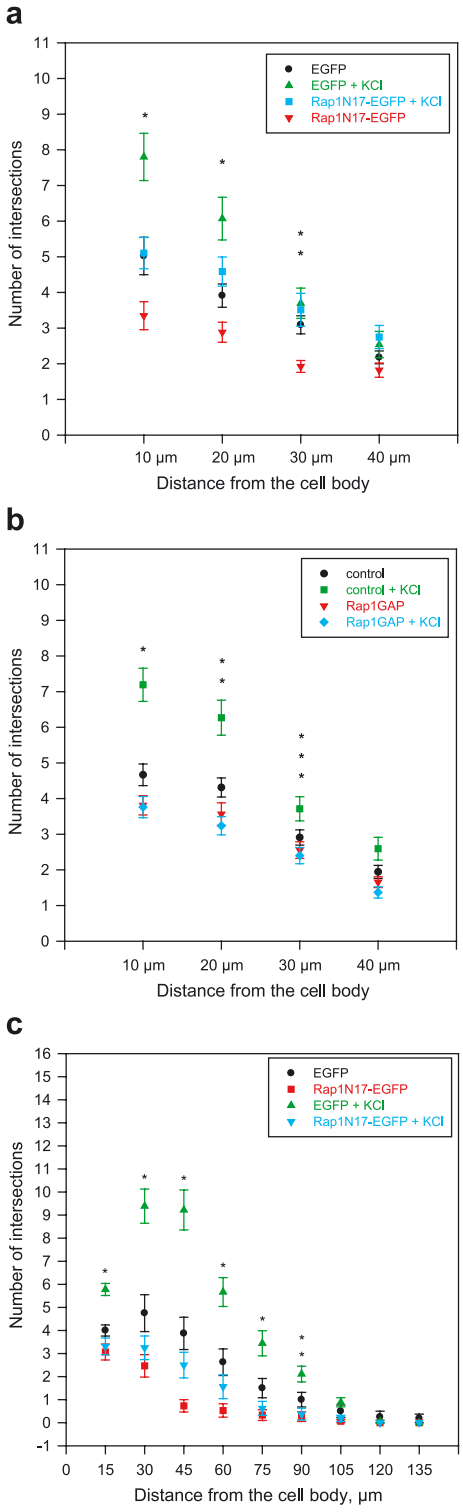
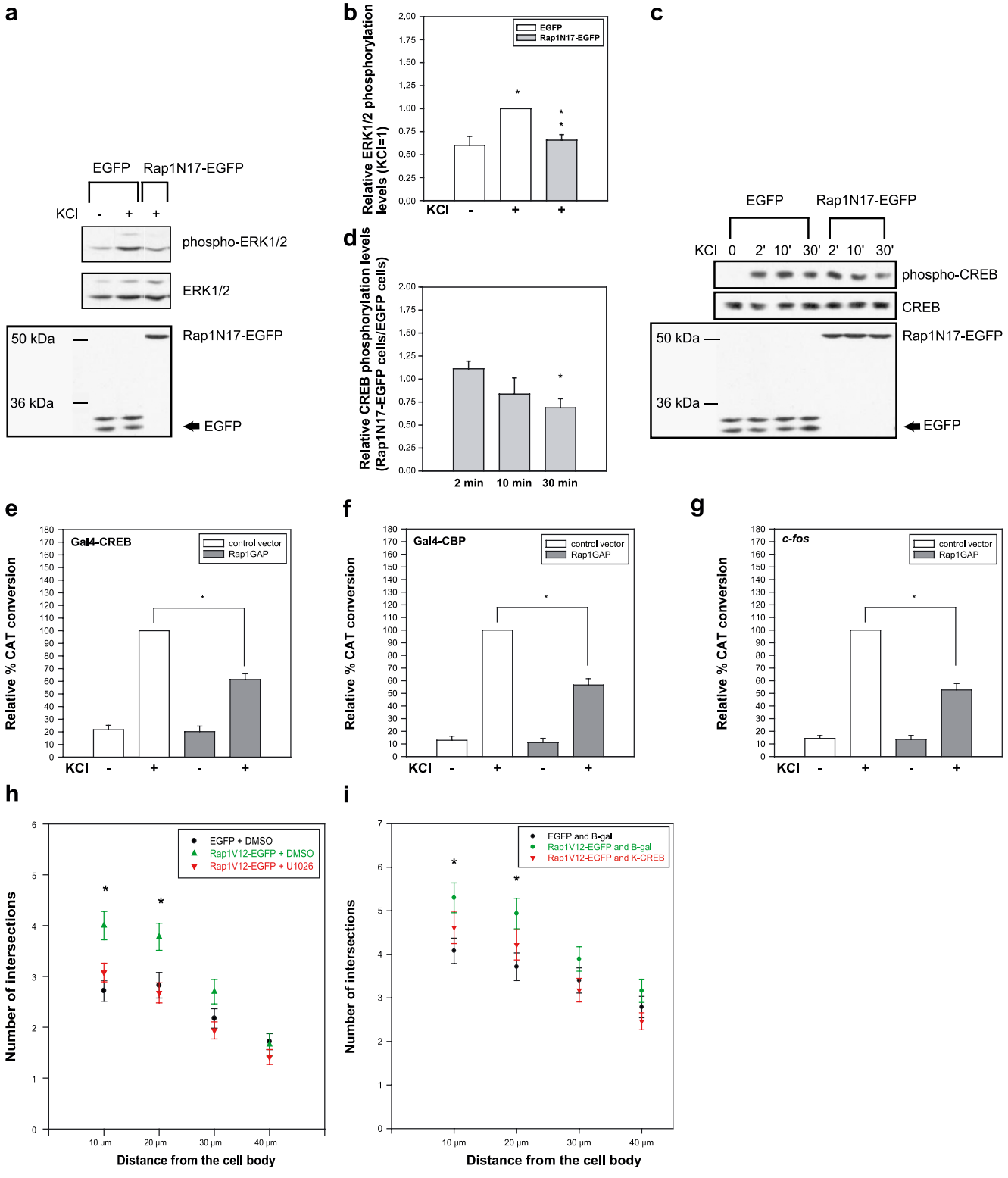


Fig. 5. Depolarization-induced dendritic growth and branching requires Rap1 function. (a) E18 cortical neurons were transfected with a control plasmid encoding EGFP or the Rap1N17-EGFP expression construct. Transfected neurons were left untreated or treated with 50 mM KCl, fixed and processed for immunofluorescence. The dendritic morphology of transfected neurons was analyzed. Results are presented as mean number of intersections plotted as a function of distance from the cell body (center = 0) to 40 µm distance. Typically, 15–20 neurons were analyzed per condition within each experiment. Data from three independent experiments are shown as mean ± SEM. Single asterisks indicate statistical significance ($p < 0.05$) by Student's t test for indicated pairs: EGFP vs. Rap1N17-EGFP, EGFP vs. EGFP + KCl, and EGFP + KCl vs. Rap1N17-EGFP + KCl at 10 and 20 µm distances. Double asterisk indicates statistical significance between EGFP-expressing cells and Rap1N17-EGFP-expressing cells at 30 µm distance. (b) E18 cortical neurons were transfected with a vector containing EGFP (control) or an expression construct encoding both EGFP and Rap1GAP (Rap1GAP). Neurons were left unstimulated or stimulated with KCl and processed for immunofluorescence. Subsequently, the dendritic morphology of transfected neurons was analyzed. Within each experiment, 15–20 neurons were analyzed per condition. Data from three independent experiments are shown as mean ± SEM. Single asterisk indicates statistical significance ($p < 0.05$) for indicated pairs: control vs. Rap1GAP, control vs. control + KCl, control + KCl vs. Rap1GAP + KCl, and control vs. Rap1GAP + KCl at 10 µm distance. Double asterisk indicates statistical significance for indicated pairs: control vs. control + KCl, control + KCl vs. Rap1GAP + KCl, and control vs. Rap1GAP + KCl at 20 µm distance. Triple asterisk indicates statistical significance for control + KCl vs. Rap1GAP + KCl at 30 µm distance. (c) Layer 5 pyramidal neurons expressing EGFP or Rap1N17-EGFP were unstimulated or stimulated with KCl. The entire basal dendritic trees of neurons were analyzed by Scholl analysis. Image stacks of a total of 15–18 neurons per condition collected from 3 independent experiments were analyzed. Results are presented as mean number of intersections plotted as a function of distance from the cell body (center = 0). Single asterisks indicate statistical significance ($p < 0.05$) by Student's t test for indicated pairs: EGFP vs. Rap1N17-EGFP, EGFP vs. EGFP + KCl, and EGFP + KCl vs. Rap1N17-EGFP + KCl at 15–75 µm distances. Double asterisk indicates statistical significance for indicated pairs: EGFP vs. EGFP + KCl, and EGFP + KCl vs. Rap1N17-EGFP + KCl at 90 µm distance.

calcium induction of the c-*fos* promoter. Our results showed that Rap1GAP expression significantly suppressed KCl-induced c-*fos* promoter activity (Fig. 6g). Thus, Rap1 signaling contributes to calcium induction of CREB-dependent promoters in cortical neurons.

To determine if the ability of Rap1 to regulate dendritic development involves ERK activation and CREB-dependent transcription, we investigated the effect of inhibiting ERK1/2 and CREB function on Rap1V12-induced dendritic growth. We determined the effect of U0126, which prevents ERK activation by MAPK kinase (MEK), on dendritic growth in Rap1V12 expressing neurons. As shown in Fig. 6h, U0126 inhibited dendritic growth induced by Rap1V12. KCREB contains a mutated amino acid within the DNA-binding domain and acts as a dominant



repressor of CREB. We also determined the effect of KCREB expression on the ability of Rap1V12 to induce dendritic growth. Whereas expression of KCREB does not affect basal dendritic growth (Redmond et al., 2002), expression of KCREB reduced Rap1V12-EGFP-induced dendritic growth by approximately 40%, indicating that Rap1 regulation of dendritic development requires CREB function (Fig. 6i). Taken together, our results demonstrate that depolarization regulates dendritic growth by a Rap1-dependent mechanism, and that the effects of Rap1 are mediated by ERK1/2 and, at least in part, by CREB-dependent transcription.

Discussion

Our results identify Rap1 as a critical regulator of calcium/activity-dependent dendritic growth in cortical neurons. The finding that Rap1 regulates dendritic development extends prior observations implicating Rap1 function in morphological regulation in various cell types. In PC12 cells, Rap1 is required for neurite outgrowth (Anneren et al., 2000; Lu et al., 2000). In *Drosophila*, Rap1 regulates normal morphogenesis of embryos and cell shape (Asha et al., 1999). In hippocampal neurons, Rap1 activity has been implicated in the formation of filopodia on the dendritic shafts and the Rap1GAP, SPAR, causes spine head enlargement, potentially by inhibiting Rap1 signaling in filopodia (Pak et al., 2001).

Based on the previous and present results, we propose a model to illustrate how Rap1 might function in calcium regulation of dendritic growth (Fig. 7). In this model, depolarization-induced calcium influx triggers the nNOS/NO/sGC/cGMP/PKG- and cAMP-dependent signaling cascades resulting in Rap1 activation. This cGMP- and cAMP-mediated activation of Rap1 is essential for calcium regulation of dendrite morphogenesis (Y.C. and A.G., unpublished observations). Furthermore, Rap1 is involved in calcium activation of ERK1/2, CREB and CBP in cortical neurons. Calcium-induced dendritic growth has been shown to be mediated by CREB/CBP-dependent transcription (Redmond et al., 2002). Results presented in this study indicate that Rap1 regulation of dendritic development via CREB-mediated transcription.

We find that calcium influx through NMDA receptors and L-type VSCCs leads to Rap1 activation. The dual involvement of both NMDA receptors and VSCCs in depolarization induced Rap1 activation may represent a unique property of Rap1. In contrast to the sustained activation seen with depolarization, glutamate activation of NMDA receptors leads to a transient activation of Rap1, that is, returning to near basal levels by 10 min (Supplementary Fig. 2). It is possible that signaling pathways that couple NMDA receptors to Rap1 activation are responsible for the initial activation of Rap1, whereas those linking VSCCs to Rap1 are important for sustaining Rap1 activity following its initial induction. Optimal levels of Rap1 signaling therefore require both NMDA receptors and VSCCs.

Upon entering the cell, calcium can activate Rap1 via several mechanisms. Calcium can directly bind to the Rap1GEF, CalDAG-GEFI, which activates Rap1. Also, Rap1 can be activated by cAMP binding to cAMP-GEF/Epac or PKA. Here, we identify another pathway involving nNOS, sGC, cGMP and PKG by which calcium influx can regulate Rap1 activation. Our data are in line with previous observations showing Rap1 phosphorylation in response to NO (Grunberg et al., 1995) and by PKG *in vitro* (Miura et al., 1992), as well as NO induction of neurite formation in E17 mouse hippocampal neurons co-cultured with astrocytes (Hindley et al., 1997). Although the precise mechanism by which cGMP signaling regulates Rap1 activation is not known, it is noteworthy that phosphorylation by PKG has been reported to be required for full activation of Rap1 (Scheele et al., 1998).

Our results provide further evidence that Rap1 is involved in calcium signaling via ERK1/2 to CREB and CBP in cortical neurons. Although the ability of Rap1 to regulate ERK signaling has been studied in other systems, whether Rap1 activates or inhibits ERK signaling appears to depend on cell type and specific experimental conditions (Stork, 2003). For example, in hippocampal neurons Rap1 is not involved in depolarization-induced ERK activation (Wu et al., 2001c). However, in PC12 cells Rap1 mediates calcium induction of Elk1-dependent transcription implicating a role of Rap1 in calcium activation of ERK (Grewal et al., 2000a). Here, we show that in cortical neurons, Rap1 is involved in calcium activation of ERK1/2. Previous studies have

Fig. 6. Rap1 is involved in depolarization-induced ERK1/2 phosphorylation, CREB/CBP-dependent transcription and c-*fos* expression. (a and b) Phosphorylated and total ERK1/2 levels in EGFP- and Rap1N17-EGFP-expressing cells following 10-min KCl stimulation were measured by immunoblot analysis. Results from 3 different experiments are presented in (b). The level of ERK1/2 phosphorylation in KCl-treated EGFP-expressing cells was set as 1. Single asterisk indicates statistical significance ($p < 0.05$) between untreated EGFP-expressing cells and KCl-treated EGFP-expressing cells by Student's *t* test. Double asterisk indicates statistical significance between KCl-treated EGFP-expressing cells and KCl-treated Rap1N17-EGFP-expressing cells. (c and d) The levels of phosphorylated and total CREB were determined in EGFP-expressing cells and Rap1N17-EGFP-expressing cells before KCl addition (0 min) and at 2, 10, and 30 min following KCl stimulation by immunoblot analysis. Results from three different experiments are shown in (d). The levels of phospho-CREB were first normalized against total CREB levels. The normalized value for Rap1N17-EGFP-expressing cells was compared to that of EGFP-expressing cells at individual time point and shown as "relative" CREB phosphorylation levels. Asterisk indicates statistical significance ($p < 0.05$) comparing 30 min to 2 min by Student's *t* test. For (a) and (c), shown are representative blots from multiple experiments. Blots were also stripped and reprobed with anti-GFP antibodies to detect EGFP and Rap1N17-EGFP expression. (e–g) Neurons were co-transfected with the parent vector (control vector) or Rap1GAP encoding plasmid (Rap1GAP), Gal4-CREB (in e) or Gal4-CBP (in f), and a reporter construct containing the Gal4 binding site, UAS-CAT. In (g), cells were co-transfected with the parent vector (control vector) or Rap1GAP encoding plasmid (Rap1GAP), and a reporter construct containing c-*fos* promoter fused to CAT (pFosCAT). One day after transfection, cells were treated with 50 mM KCl. Cells were lysed 16–20 h following KCl treatment and assayed for CAT activity. Each experiment was performed three times independently. The percent of CAT conversion in KCl-stimulated EGFP-expressing cells was set as 100%. Data are presented as means of relative % CAT conversion ± SEM. Asterisk indicates statistical significance ($p < 0.05$) between KCl-treated EGFP-expressing cells and KCl-treated Rap1GAP-expressing cells by Student's *t* test. (h) Neurons were transfected with EGFP or Rap1V12-EGFP expression construct. EGFP transfected neurons were treated with DMSO and Rap1V12-EGFP transfected neurons were treated with DMSO or U0126. Results of Scholl analysis are shown. Asterisks indicate statistical significance ($p < 0.05$) for the indicated pairs: EGFP + DMSO vs. Rap1V12-EGFP + DMSO, Rap1V12-EGFP + DMSO vs. Rap1V12-EGFP + U0126. (i) Neurons were co-transfected with EGFP or Rap1V12-EGFP expression construct along with β-gal- or KCREB-encoding plasmid. The dendritic branches of neurons were analyzed by Scholl analysis. Asterisks indicate statistical significance ($p < 0.05$) for the indicated pairs; EGFP + β-gal vs. Rap1V12-EGFP + β-gal, Rap1V12-EGFP + β-gal vs. Rap1V12-EGFP + KCREB.

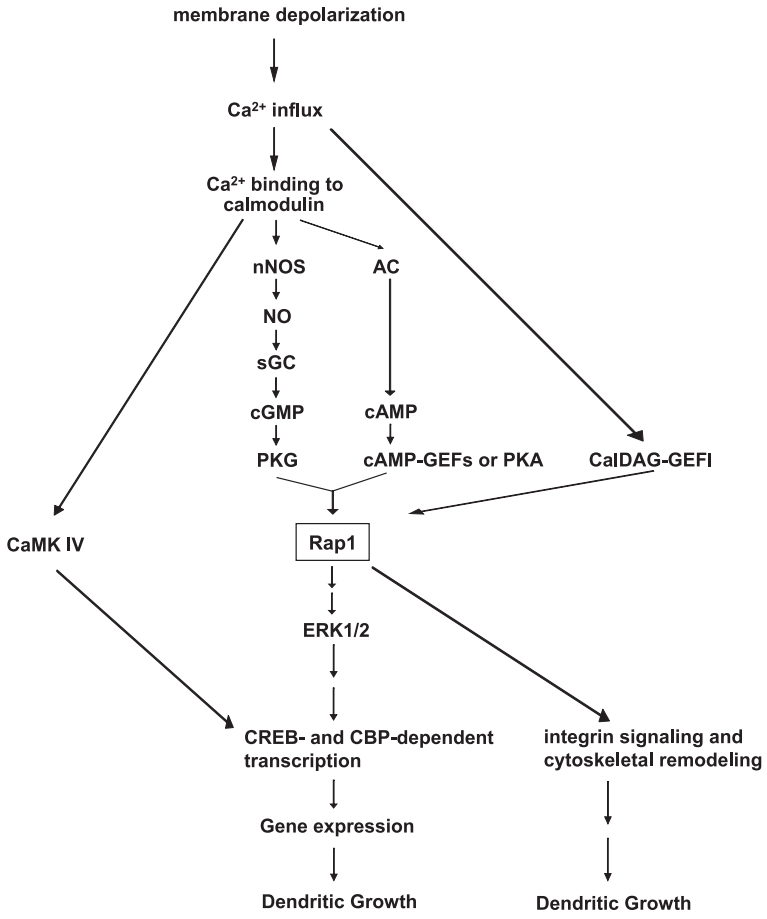


Fig. 7. A model depicting the proposed role of Rap1 in calcium regulation of dendritic growth. See text for details. Double arrows imply that intermediate steps are involved.

emphasized the role of Ras in calcium activation of ERKs (Rosen et al., 1994; Dolmetsch et al., 2001). Based on our results, we consider it likely that both Ras and Rap1 contribute to ERK activation in response to calcium influx, as it is the case with NGF signaling. In PC12 cells, Ras is required for the initial activation of ERK in response to NGF, and Rap1 acts by sustaining ERK activation (York et al., 1998). Together, Ras and Rap1 contribute to NGF-dependent ERK activation and neuronal differentiation. Therefore, in response to calcium entry, Ras may similarly initiate the early phase of ERK activation and Rap1 may mediate sustained ERK activation. It remains to be determined whether Ras contributes to depolarization-induced dendritic growth. We also find that Rap1 is involved in calcium induction of CREB/CBP-dependent transcription and c-*fos* expression. The ability of Rap1 to regulate CREB- and CBP-dependent transcription supports a mechanism by which Rap1 may control the expression of genes required for dendrite morphogenesis.

Previous work from our laboratory demonstrates that CaMK IV is involved in depolarization induced dendritic growth (Redmond et al., 2002). Since dominant repressor of CREB (KCREB) inhibits dendritic growth induced by constitutively active forms of Rap1 and CaMK IV, it appears that both Rap1 and CaMK IV regulate dendritic development via CREB-dependent transcription. To examine whether there is a crosstalk between Rap1 and CaMK IV signaling, we determined the effect of expressing a kinase-dead version of CaMK IV (CaMK IV K75E) as well as the CaMK IV inhibitor KN62 on depolarization induced Rap1 activity. While CaMK IV K75E did not appear to affect KCl induced Rap1 activity in our preliminary experiments, we found that KN62 inhibited KCl induced Rap1 activity by approximately 30% (data not shown). However, given the effect of KN62 on calcium influx and another CaMK, CaMK II, it remains unclear as to how CaMK IV and Rap1 signaling pathways are integrated. While CaMK IV has been implicated in rapid activation of CREB following depolarization (Dolmetsch et al., 2001; Ho et al., 2000; Ribar et al., 2000; Wu et al., 2001b), Rap1 has been shown to act by sustaining the activation of ERK which regulates CREB (York et al., 1998). Thus, CaMK IV may mediate the rapid activation of CREB, and Rap1 may sustain or prolong the signal leading to CREB-dependent transcription. Together, CaMK IV and Rap1 contribute to depolarization-induced dendritic growth via a CREB-dependent mechanism.

While CREB is necessary for calcium regulation of dendrite formation (Redmond et al., 2002; Vaillant et al., 2002; Wu et al., 2001c), some of the effects of Rap1 may be mediated by CREB-independent mechanisms. This possibility is supported by our results showing that KCREB did not completely inhibit Rap1V12-induced dendritic growth. Furthermore, Rap1GAP expression abolished the ability of KCl to induce dendritic growth without completely inhibiting CREB-dependent transcription. One potential mediator of the CREB-independent effect on dendritic development is integrin.

Integrins are heterodimeric receptors that function in cell adhesion to the extracellular matrix (ECM). Integrin signaling has been implicated in neurite outgrowth (Clegg, 2000) and this can probably be attributed to the fact that neuronal processes need to maintain strong attachments to the underlying ECM to become stabilized and to prevent retraction. In retinal neurons, α6β1 integrin has been shown to promote neurite outgrowth on laminin-1 (Ivins et al., 2000). In cerebellar granule neurons, α7β1 integrin is involved in neurite outgrowth induced by tenascin-C and laminin-1 (Mercado et al., 2004). Since there is compelling evidence that Rap1 controls cell adhesion by regulating the ligand-binding ability of integrins in non-neuronal cells (Caron, 2003), Rap1 regulation of integrin activation might represent a mechanism for dendrite morphogenesis that is independent of CaMK IV and CREB (Fig. 7). It is intriguing that Rap1 modulation of integrins' ability to bind ligands in ECM may be particularly important in stabilizing dendrites thereby preventing the pre-existing dendrites from retracting and promoting the newly formed dendrites to adhere to ECM as they continue to grow. We show that Rap1V12 induces dendritic growth without concurrent CaMK IV activation (Fig. 2). One possible explanation for this result is that constitutive Rap1 signaling prolongs integrin activation and enhances the adhesion of pre-existing and newly formed dendrites to ECM thereby stabilizing dendritic arbors in Rap1V12 expressing neurons.

Taken together, Rap1 appears to contribute to calcium-regulated dendritic development via both CREB/CBP-dependent and-independent mechanisms (Fig. 7). The ability of Rap1 to regulate CREB/CBP-dependent transcription in the nucleus would enable Rap1 to control the expression of genes required for dendritic growth. In addition, Rap1 activation could modulate integrin receptor function and stabilize dendritic arbors. The effects of Rap1 on neuronal morphology support an important role of Rap1 signaling in the development of cortical circuits.

Experimental methods

Primary cell cultures

Dissociated cortical cultures were prepared from the cortices of E18 Long-Evans rats as previously described (Ghosh and Greenberg, 1995; Threadgill et al., 1997). Cells were routinely grown in L-glutamine-free Basal Media Eagle Media (BMEM) supplemented with 1% N2 supplement, 1 mM L-glutamine, 5% fetal bovine serum (FBS), 1 mM penicillin-streptomycin. All reagents were purchased from Invitrogen (San Diego, CA).

DNA constructs and pharmacological compounds

Rap1N17 and Rap1V12 cloned into pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) were gifts of Dr. V. Pizon. The Rap1V12 mutant contains a Gly to Val substitution at position 12. This substitution reduces the intrinsic GTPase activity of Rap1 and consequently gives rise to a constitutively GTP-bound protein. The Rap1N17 mutant contains a Ser to Asn substitution at codon 17 thereby increasing its affinity for GDP. Rap1N17 acts in a dominant negative fashion by sequestering endogenous RapGEFs making them unavailable for the activation of Rap. The Rap1GAP cDNA (Polakis et al., 1991) in pMT2-HA and GST-RalGDS-RBD cDNA were generously provided by Dr. J. L. Bos. The Rap1GAP cDNA was later subcloned into the *Bam*H1 and *Eco*RI sites of pBMN-IRES-EGFP (provided by Dr. G. P. Nolan). Neurons transfected with this construct express both EGFP and Rap1GAP proteins. The following plasmids have been described previously: Gal4-CBP (Chrivia et al., 1993; Swope et al., 1996) (full-length), UAS-CAT (Martin et al., 1990) and pFosCAT (−359 to +12 of mouse c-*fos* promoter) (Chen et al., 1999). Inhibitors were used at the following concentrations: L-NAME (1 mM, Sigma N5751, St. Louis, MO), ODQ (1H-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one, dissolved in DMSO, 1 μM, Sigma 03636), SQ22536 (300 μM, Sigma S513), Rp-8-pCPT-cGMP TEA salt (100 μM, Calbiochem 370677, San Diego, CA), nifedipine (dissolved in DMSO, 20 μM, Sigma N7634), D-(−)-2-amino-5-phosphonopentanoic acid or APV (100 μM, Calbiochem 165304), EGTA (2 mM, Sigma E4378), U0126 (25 μM, Promega) and SB203580 (dissolved in DMSO, 10 μM, Calbiochem 559398). L-glutamic acid (100 μM, monosodium salt, Sigma) and 8-pCPT-cGMP (400 μM, Calbiochem) were also used in this study.

Transfection

For dendrite studies, dissociated E18 neurons (10^6 cells per well) were plated on 18 mm polylysine-laminin-coated glass cover slips placed in the wells of 12-well plates. One to 2 h after plating, cells were transfected using a calcium-phosphate-based transfection procedure as previously described (Threadgill et al., 1997). Four micrograms of DNA per well was used. KCl was added to the media 20–24 h post-transfection to a final concentration of 50 mM. For KCREB experiments, cells were transfected with 3 μg of Rap1V12-EGFP containing plasmid along with 6 μg of KCREB or β-gal encoding construct. For determining the effect of U0126 on dendritic growth in Rap1V12-EGFP expressing neurons, U1026 was added 1 day after transfection. Cells were fixed 2 days post-transfection. Cells were fixed at 2 days in vitro (DIV). For the analysis of phospho-ERK1/2 and phospho-CREB levels, cells (5×10^6 cells per dish) were plated on polylysine-laminin-coated 60-mm dishes. Cells were transfected at 2 DIV with 5 μg of plasmid DNA per plate using Lipofectamine 2000 (Invitrogen) according to manufacturer's suggestions. One day post-transfection, cells were switched to low serum condition by diluting the condition media at 1:10 in L-glutamine supplemented BMEM. Cells were stimulated with 50 mM KCl and lysed for immunoblot analysis 2 days after transfection. For CAT assays, neurons (10^6 cells per well) plated on polylysine-laminin-coated 12-well plates were transfected at 3 DIV using Lipofectamine 2000. For CREB-mediated transcription, cells were co-transfected with 0.1 μg of Gal4-CREB and 0.1 μg of UAS-CAT along with 1 μg of the parent vector or Rap1GAP encoding plasmid per well. For CBP-dependent transcription, cells were co-transfected with 0.5 μg of Gal4-CBP and 0.5 μg of UAS-CAT along with 1.5 μg of the parent vector or Rap1GAP encoding plasmid per well. For c-*fos* promoter activity, cells were co-transfected with 0.1 μg of pFosCAT reporter along with 1 μg of the parent vector or Rap1GAP encoding plasmid per well. The total amount of DNA per well was maintained constant within each experiment.

Immunofluorescence studies

For studies on the subcellular localization of Rap1, E18 cortical neurons were fixed 18–24 h after plating. The rabbit polyclonal anti-Rap1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz,

CA) was used at 1:10 and immunofluorescence studies were performed as described (Polleux et al., 2000). Immunofluorescence analysis using the mouse monoclonal anti-Rap1 antibody (1:300, BD Transduction Laboratories, San Diego, CA) was performed as described (Wu et al., 2001a). For dendrite studies, immunofluorescence studies were performed as previously described (Polleux et al., 2000). Transfected neurons were double labeled with rabbit polyclonal anti-GFP antibodies (1:3000, Molecular Probes, Eugene, OR) to outline neuronal processes and mouse anti-MAP2 antibodies (1:3000, Sigma) to identify dendrites. The fluorophore-conjugated secondary antibodies used were Alexa Fluor 568 goat anti-mouse IgG (2 μg/ml, Molecular Probes) and Alexa Fluor 488 goat anti-rabbit IgG (2 μg/ml, Molecular Probes). Since nuclei of apoptotic cells undergo shrinkage and fragmentation, we also counter stained cells with Höechst (Sigma), a DNA dye, to reveal nuclear morphology, and to demonstrate that cells included in dendrite analysis contain intact nuclei.

Scholl analysis of dendrites of dissociated neurons

Two days post-transfection, cells were processed for GFP/MAP2/Höechst staining (see above for details). Images of transfected neurons (GFP and MAP2 positive with intact nuclei) were captured without any prior knowledge of the experimental condition. Only neurons with identifiable axons were included in the analysis. Axons were defined as the longest process of all extended from the cell body exhibiting a taper appearance and maintaining about the same thickness through out the entire length. Moreover, axons stained negative for MAP2 except in the region very proximal to the cell body. These features are reminiscent of those of neurons that have reached the final position in the cortical plate or the cell dense zone just beneath the molecular layer *in vivo*. Images of randomly chosen neurons were acquired at 40X magnification using a Nikon inverted microscope equipped with Openlab 3.0 software (Improvision, Lexington, MA). Typically, images of 15–20 neurons per condition were captured for each independent experiment. Since each experiment was performed no less than three times independently, this generally represents a total of 45–60 neurons per condition from three separate experiments. Acquired images were analyzed with Openlab 3.0 software. To provide a quantitative measure of dendritic branching pattern, the number of dendrites that cross concentric circles spaced 10 μm apart starting at the center of the cell body was counted.

Cortical slice cultures and ballistic-based transfection method

Coronal sections (350 μm) were obtained from cortices of postnatal day 5 (P5) rats and cultured as previously described (Redmond et al., 2002). At 1 DIV, slices were transfected ballistically with plasmid DNAs coated onto 1.6 μm gold particles. Slices were bombarded with a gene gun (Helios Gene Gun, Bio-Rad, Hercules, CA) using 125 psi. At 2 DIV, slices were left untreated or treated with 50 mM KCl for 19–21 h. At 3 DIV, slices were fixed and processed for GFP/MAP2/Höechst immunofluorescence studies. Layer 5 of the cortex was identified based on Höechst and MAP2 immunostaining. Images of z sections of the entire basal dendritic trees of layer 5 pyramidal neurons were captured by Perkin-Elmer UltraView confocal microscope (Perkin-Elmer, Boston, MA). Basal dendrites of neurons were traced through the entire image stacks. The number of basal dendrites that intersect a series of concentric circles spaced 15 μm apart starting at the center of the cell body was counted.

Rap1 activation assay

Cells (5×10^6 per dish) were plated on polylysine-laminin-coated 60-mm dishes. Two or 3 days after plating, cells were switched to low serum growth media prepared by diluting the condition media at 1:10 with BMEM supplemented with 1 mM L-glutamine. On the following day, cells were stimulated with 50 mM KCl. Cells were lysed immediately following stimulation to determine the activation status of Rap1 as previously described (Franke et al., 1997). Briefly, each plate of cells was lysed in 500 μl of 1× lysis buffer. Following lysis, a fraction of extract was saved for the analysis of total Rap1 protein levels. An amount of lysates corresponding to 400 μg of total protein was mixed with 10 μg of glutathione-agarose coupled GST-RalGDS-RBD for pulling down the GTP-bound form of Rap1 in total cell lysate. Rap1 was detected by immunoblot analysis using a rabbit anti-Rap1 antibody (1:100, Santa Cruz). This is a non-radioactive method for measuring the amount of Rap1-GTP in cell lysates. For determining the effect of various inhibitors on Rap1 activation status, before KCl stimulation (10 min) cells were pretreated with EGTA for 1 min, L-NAME or SB203580 for 1 h, or other inhibitors for 30 min.

Immunoblotting

To measure the amount of phosphorylated ERK1/2 and CREB proteins using phospho-specific ERK1/2 and CREB antibodies (Cell Signaling Technology or CST, Beverly, MA), cell lysates were prepared according to the manufacturer's protocol. Typically, 200 μl of 1× SDS sample buffer were used to lyse cells in a 60-mm dish. Blots were probed with the following CST antibodies at 1:1000 dilutions: rabbit anti-p44/42 MAP Kinase, mouse anti-phospho-p44/42 MAP Kinase, rabbit anti-CREB and rabbit anti-phospho-CREB (Ser133). Bands were visualized by enhanced chemiluminescence (SuperSignal® West Pico Substrate, Pierce Biotechnology, Rockford, IL). The intensity of bands on autoradiograms of immunoblots was determined by using Personal Densitometer SI (Amersham, Piscataway, NJ) and the analysis software ImageQuant (Amersham).

CAT Assay

Following transfection, cells were switched to low serum media prepared by diluting the condition media at 1:20 in L-glutamine supplemented BMEM. Cells were stimulated with 50 mM KCl 22–24 h post-transfection and harvested 16–20 h following KCl stimulation for CAT assay. Total CAT activity was measured as previously described (Talmage and Listerud, 1994) using an amount of extract that maintain activity in a linear range, that is, 1–50% of substrate conversion. Each experiment was performed no less than three times independently.

Statistical analysis

The results were analyzed for statistical significance using SigmaPlot (SPSS Inc., Chicago, IL). Statistically significant differences between control and experimental conditions are defined as $p < 0.05$ by Student's t test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2004.08.012.

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